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Induced Multiple Ovulation in the Bovine

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INDUCED MULTIPLE OVULATION IN THE BOVINE

BY

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INDUCED MULTIPLE OVULATION IN THE BOVINE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Major Adviser Date

Head, Animal Science Department Date

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To the cow-calf operator, the single most important factor affecting his economic livelihood is the number of calves produced and weaned for a given year. Unfortunately, due to low reproductive efficiency in comparison with other cattle traits, the possibility of having a calf crop of greater than 80 percent at weaning is not too common. The low heritability estimates that have been reported for reproductive performance indicate that little improvement can be expected through selection.

To the animal scientist this problem can best be met by the development of a simple and reliable method of inducing for the most part, twins in cows through the use of gonadotropic hormones. The production of a calf crop topping 100 percent and possibly even above 150 percent, similar in comparison to a natural lamb crop, could be expected. The economic advantages of induced twinning can best be utilized in cows producing calves for beef production.

From the standpoint of whether or not a beef cow can efficiently suckle twin or triplet calves can probably best be decided by the milk producing abilities of the individual dam. However, it can be assumed that crossbreeding and especially the crossing of breeds of beef and dairy together will provide an adequate milk supply. If the contrary is true, rearing the multiple calves artificially as the dairy people have been doing for a number of years would be a possible solution.

INTRODUCTION

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The practical use of induced twinning in the bovine at the farm or ranch level is yet to be seen; however, with the advances being made with estrus synchronization and studies pertaining to the control of ovulation, it seems quite likely that its future use will become a reality. At the present time it seems quite unlikely that the induction of twin pregnancy will lend itself to practical application where large herds of beef cattle are managed under range conditions.

The combination of a successful estrus synchronization program with a gonadotropin treatment resulting in a large share of cows responding with twin pregnancies would have a great effect upon the cattle industry. It is with this last goal in mind, that research continues to find the most desirable hormonal injection or sequence of injections that will stimulate a consistent number of limited ovulations (two or three) which will result in a high percentage of twin births without lowering the subsequent fertility of the cow herd.

The purpose of this experiment was to study and compare the ovulatory response between a group of beef heifers receiving a single gonadotropin injection in the follicular phase of the estrous cycle with a group receiving two injections of the same gonadotropin: one in the luteal phase and the other in the follicular phase.

REVIEW OF LITERATURE

Spontaneous Multiple Births

Incidence. In the bovine, as well as with many other monotocous ungulates, spontaneous multiple pregnancies do occur quite commonly. There is a considerable amount of information in the literature dealing with the incidence of multiple births in cattle. The occurrence of twins is fairly common, but only rarely do cows give birth to triplets and quadruplets, though cases are on record where triplets and quadruplets have been born and reared. The incidence of multiple births may vary within breeds, between breeds, with age of animal, with season or with the environment under which cattle are maintained.

The frequency of cattle giving birth to multiples is generally considered to be less than 5.0 percent. Richter (1955) examined data of over 59,000 calvings and found the frequency of multiple births to be 3.2%, of which 0.01% were triplets and 0.002% quadruplets. The incidence of multiple births varies considerably among breeds and is known to be much higher in dairy cattle than in beef cattle. Johansson (1932) gives 1.88 and 0.44 as the incidence of twin births in dairy and beef cattle, respectively. Twinning occurred with an incidence of approximately 0.5% out of 8857 Hereford births at a U.S. range livestock experiment station (Woodward and Clark, 1959). Hewitt (1934) gives the incidence as 2.0% in Red Polls and 3.0% in Friesians. In 7,387 calvings in a Holstein-Friesian herd covering a 30 year period, 338 or 4.58% were multiple births, of which two were triplets (Erb and Morrison, 1959). Combining their own data on 10,885 calvings with that of numerous other authors, Meadows and Lush (1957) found a total twinning incidence of 2.58% in the five major dairy breeds in the United States. Among dairy breeds, twins are most frequent in Holsteins and least common in Jerseys (Lush, 1925; Meadows and Lush, 1957). Joubert (1961) reported that European breeds of cattle will generally have a higher frequency of multiple births than African breeds.

Types of Twins. Dizygotic twins (fraternal) are produced from two ova. Each ovum represents a different sample of maternal germ plasm that is fertilized by a different sperm. Each twin, therefore, is the result of a different random sampling of the paternal and maternal germ plasm. Fraternal twins of like and unlike sex would be expected to occur in a sex ratio consisting of: 1 set of male twins; 2 sets of unlike twins (male and female); 1 set of female twins. From data on 9,441 pairs of twins, Johansson and Venge (1951) reported that 26% were both males, 47% were one male and female, and 27% were both females.

Monozygotic (identical) twins are produced from the cleavage of a single zygote. Both are the same sex and possess the same genetic material; therefore, they are genotypically alike. The incidence of monozygotic twins among like-sexed pairs was found to be approximately 8.6% by Meadows and Lush (1957). Erb and Morrison (1959) estimated that 13.68% of the like-sexed twins in the Carnation Holstein herd were monozygotic. These monozygotic twins represented 7.4% of all twins and 0.3% of all births.

Hereditary Influence. The variation in frequency of multiple births between herds, cow families, sire groups and breeds provides evidence that heredity plays some role in the incidence of multiple births in cattle (Lush, 1925; Erb *et al.*, 1960). Pfau *et al.* (1948) have shown a uniform positive trend between the incidence of twinning and level of inbreeding in Holstein-Friesian cattle. Somewhat contrary to the evidence that twinning increases with inbreeding is the increased rate of multiple ovulations found in outbred cattle over inbreds (Labhsetwar *et al.*, 1963a). Heeren (1957) states that twinning is often a heritable characteristic and concludes that it is caused by a recessive gene affecting the 'X' chromosome. Although there is considerable evidence that the occurrence of two egg twins is to some extent inherited, the same does not hold true for one egg twins (Donald and Anderson, 1953). They found one set of two-egg twins from 122 pregnancies of one-egg twin cows mated to one-egg twin bulls. No twins were obtained from 60 pregnancies of two-egg twin cows mated to one-egg twin bulls. They concluded that one-egg twinning is a sporadic event provoked by uterine conditions from fertilized eggs not genetically predisposed to it.

General. Multiple births are rare at first parturition, rise to a peak at the fifth to seventh parturitions and then decrease thereafter (Hewitt, 1934; Pfau *et al.*, 1948; Erb and Morrison, 1959). The above statement is in close harmony with the general cycle of fecundity in most multiparous mammals; therefore, twinning may be possibly an expression of fecundity or the potential reproductive capacity of a cow (Pfau et al., 1948).

Various authors have reported a high incidence of multiple births in cows which conceived upon recovery from cystic ovaries (Clapp, 1934; Erb et al., 1960). Clapp (1934) suggested that twinning may result from cystic ovaries, which is a pathological condition of common occurrence in high producing cows after their first calving. In support, Labhsetwar et al. (1963a) found that the incidence of multiple ovulations in cystic parities was about three times as high as in noncystic ones. Erb et al. (1960) found that 71 percent of 266 twinning cows also had a tendency towards cystic ovaries in one or more reproductive periods and these same cows produced 74 percent of the multiple births.

As evidenced by the literature on natural twinning, the desirability of twinning in cattle is still controversial. Erb and Morrison (1959) presented evidence indicating that twin cows reproduce as well as their singly born herd mates; however, cows producing twins at one of the first three calvings had fewer subsequent calvings than did cows producing singles. The influence of cows twinning on their subsequent reproduction has been that cows have longer calving intervals and require more services for conception (Pfau et al., 1948). Wilson (1955) found that after 70 cows gave birth to single calves, they required 1.55 services for conception, but following the birth of twins, 1.83 services were required. The greater incidence of relative infertility after twinning was found to be due primarily to the proportionately greater number of post-parturient disorders as compared with single births (Erb and Morrison, 1959). The retention of placental membranes following twinning has been reported to occur about three times as often as in cows producing singles (Pfau *et al.*, 1948; Erb *et al.*, 1958). These same authors report a higher incidence of abortions and stillbirths with twinning. Woodward and Clark (1959) reported the total incidence of stillbirths in beef cattle for a period of 22 years to be 3.6%; 11 (16%) of the 70 twin calves born over that period were found to be stillborn. Among twins the incidence of retained placenta and stillbirths has been found to occur significantly more often with male births (Erb *et al.*, 1958; Woodward and Clark, 1959). Comberg and Velten (1962) reported that with male twins, fetal membranes were retained 73% of the time; with mixed twins, 42%; and with female twins, only 20%. The physiological significance of these observations is yet unknown.

Hormone-Induced Multiple Ovulations

In common with other farm animals, fertility in the cow is mainly under the control of two gonadotropic hormones produced by the anterior pituitary gland: follicle stimulating hormone (FSH) which leads to the ripening of follicles in the ovaries and luteinizing hormone (LH) which brings about ovulation and the development of the corpus luteum. The balance between FSH and LH output by the pituitary varies with the different species of farm animals, and after follicular development, the level of FSH falls in favor of LH for the remainder of the estrous cycle. In the cow the tendency is for a single ovulation to occur during the normal estrous cycle; however, the incidence of spontaneous multiple ovulations in Holsteins has been found to range from a low of 5.4 percent (Labhsetwar et al., 1963a) to about 13 percent (Kidder et al., 1952). The main limiting factor concerning the occurrence of natural multiple ovulations is the amount of FSH produced by the pituitary. The pituitary output of LH is usually considered sufficient to cause ovulation of more than one ripe follicle. When exogenous FSH is administered to induce multiple ovulation, growth of individual follicles is not accelerated but more follicles are brought to maturity simultaneously.

Since Cole and Hart (1930) first demonstrated the presence of a gonadotropin similar in action to FSH in the blood of a pregnant mare, a considerable amount of research has been done with this serum gonadotropin (PMS) in the control of animal fertility. Multiple ovulation in the farm animal has been induced by various extracts of the anterior pituitary, but PMS has been used extensively due to the advantage of it being cheaper than pituitary extracts and that it is not destroyed or metabolized by the body as readily as the pituitary gonadotropins.

A large amount of literature deals with the induction of superovulation in various species by administration of gonadotropic hormones. Pincus (1940), using pituitary extracts, successfully induced superovulation in the rabbit; similar attempts using different dosage levels and given at various stages of the cycle were reported for the ewe (Hammond, Jr., et al., 1942; Murphree et al., 1944): for the sow (Tanabe et al., 1949) and for the cow (Casida et al., 1943 and Willet et al., 1948).

Hammond, Jr. and Bhattacharya (1944) were among the first researchers to use PMS for the induction of multiple ovulation in cattle. Much of the early work was concerned with inducing superovulation by administering PMS in conjunction with the enucleation of the corpus luteum. The reason for corpus enucleation was to enable PMS treatment to be independent of the estrous cycle. This was done particularly with beef animals, which are often not under such close observation as dairy cows; therefore, the problem of detecting estrus was aided by standardizing the cycle (estrus - 2 to 5 days after enucleation) for PMS injections. Where a high degree of superovulation is required for the provision of eggs for use in transplantation studies, the administration of PMS in conjunction with corpus luteum enucleation has been successful in experiments reported by a number of workers (Dowling, 1949; Rowson, 1951; Brock and Rowson, 1952; Hafez et al., 1963a).

For practical on the farm or ranch application, superovulation induced by a gonadotropin injected about the time of corpus luteum enucleation has never proved to be a satisfactory procedure. Expression of a functional corpus luteum may cause abnormal ovarian activity to occur. Foote et al. (1959) and Dawson (1961) have reported that cattle tend to develop cystic follicles and to give rise to spontaneous double ovulations following removal of the corpus luteum at the mid-cycle stage.

Administration of pituitary extracts and PMS in the immature female calf has been attempted in studies aimed at provision of eggs for transplantation and at shortening the generation interval (Casida et al., 1943; Marden, 1953; Black et al., 1953; Howe et al., 1962; Jainudeen et al., 1966). These researchers have shown that the calf is able to respond to exogenous gonadotropins very early in life and that the follicles developing as a result are capable of ovulation following an injection of LH. The major deterrent to a practical outcome is that in only a few cases have fertilized eggs been recovered.

The ovarian response to exogenous gonadotropins is dependent upon the stage of the estrous cycle at which treatment is initiated. Multiple ovulations have been obtained consistently when cows are injected either with FSH or PMS during the follicular phase of the cycle (Casida et al., 1943; Hammond and Bhattacharya, 1944; Dowling, 1949; Willet et al., 1953; Hafez et al., 1963a; Scanlon et al., 1968). The injection of a single dose of PMS 5 days prior to estrus and an intravenous injection of human chorionic gonadotropin (HCG) at either the first signs of heat or predicted time of heat (Brock and Rowson, 1952; Willet et al., 1953; Hafez et al., 1963a; Scanlon et al., 1968) has given satisfactory results.

Gonadotropin injections made during the luteal phase when an active corpus luteum is present results in very little ovulation taking place. The injection of PMS in the cow at this stage of the cycle stimulates a number of follicles to maturity but their ovulation rarely occurs unless the active corpus luteum is expressed (Folley and Malpress, 1944; Dowling, 1949; Greenstein et al., 1958). Where injection is made in the luteal phase and ovulation does take place, the percentage of eggs fertilized is very low (Casida et al., 1940, 1943; Rowson, 1951; Avery et al., 1962).

Hafez et al. (1963a) showed that multiple ovulations did not occur simultaneously in superovulated animals and that in some instances ovulations occurred over a period of 8 days. Human chorionic gonadotropin, which is luteinizing in nature and various purified pituitary extracts containing LH have long been used to stimulate ovulation; however, there still remains debate as to the necessity of exogenous LH to cause ovulation of artifically stimulated follicles in the cow. This is especially true when the spontaneous ovulatory mechanism natural to the cow is utilized with gonadotropin treatment being initiated four or five days prior to the natural occurrence of estrus.

Hormone-Induced Multiple Pregnancy

The use of gonadotropins to induce multiple ovulation resulting in the development of two or more fetuses which will successfully go to term in the cow is receiving an increased amount of study at the present time. A critical problem in gonadotropin-induced multiple pregnancies is the need to restrict the number of developing fetuses to no more than two. Stimulated cows bearing three or more fetuses are usually subject to abortion during the fifth month of pregnancy, premature births or parturition complications (Gordon *et al.*, 1962).

Dose-Response Relationship without Estrous Synchronization. There has long been known that a relationship exists between the number of ovulations and the dose of gonadotropin administered. However, one fact stands out amongst all the literature dealing with the induction of multiple ovulation in the bovine and this is that a tremendous amount of individual variation in response is found to any one standard dosage level. This variability in response has led to the study of different treatment sequences and the usage of various gonadotropins which would hopefully produce a consistent amount of limited ovulations from animal to animal. Hammond and Bhattacharya (1944) induced twin and multiple ovulations in cattle with a single subcutaneous injection of 1500 I.U. of PMS. They were among the first to artificially produce the birth of twins and triplets and to make note of the practical application of twinning especially in beef cows where the number of calves produced is most important in comparison to milk production for the dairy cow.

Hammond, Jr. (1949), in a small field trial using primarily Shorthorn cattle, reported on the birth of 13 single calves, four sets of twins and three sets of triplets. A single PMS injection of either 2000 I.U. or 2500 I.U. was used to induce multiple ovulation. Ovulatory response examined by rectal palpation showed that several cattle in the experiment did not respond with two or more ovulations. Suspicion as to the activity of the commercial PMS preparation used was checked by an assay which showed only 60% of the alleged potency.

Arbeiter (1962) gave 19 cows an intramuscular injection of 1500 I.U. PMS approximately 4 days prior to estrus; 77 percent conceived to the first insemination. Four cows had multiple births (two sets of twins, one of triplets and one of quadruplets). The hormone was said to have had a greater effect than intended and the dosage was considered too high.

Gordon et al. (1962) conducted an extensive field trial over a two year period on the induction of twin pregnancy in 525 cows predominantly of Hereford breeding. A single injection of PMS was given in the follicular phase of the estrous cycle using dosage levels of 800, 1000, 1200, 1600, and 2000 I.U. In those which exhibited estrus and ovulated after PMS treatment, the percentage of cattle producing additional eggs increased from 33 percent at 800 I.U. to 56 percent at 2000 I.U. The mean ovulation rates for the five dosage levels were 1.43, 1.77, 2.50, 2.71 and 3.97, respectively. At the 800 I.U. level, most cows that responded did so by producing two eggs only; furthermore, there was said to be a tendency for the proportion producing only twin ovulations to fall as the dosage level of PMS increased. Denny (1964) also used various dosage levels ranging from 500 to 1000 I.U. of PMS injected subcutaneously on day 17 of the estrous cycle. At the post-treatment estrus, two inseminations were made and then the cattle were slaughtered approximately 72 hours after the termination of estrus. Examination of the ovaries and recovery of the shed ova indicated that at the 1000 I.U. level PMS could be used successfully to cause the shedding of two fertilizable ova.

Hafez et al. (1964) induced multiple pregnancy in 79 nulliparous beef cattle by injecting 1500 to 2000 I.U. of PMS 16 days after the previous estrus. Response was determined by slaughter 30 to 60 days after insemination. After the PMS injection, 35 percent of the heifers did not conceive and 41 percent were carrying viable single embryos. Multiple pregnancy was observed in 24% of the cattle, of which 11 percent consisted of viable twin embryos and 13 percent included heifers bearing three to five viable embryos. Hafez et al. (1965) injected 56 beef heifers intramuscularly with 1500 I.U. of PMS on day 12 and day 16 (estrus is day 0) of the estrous cycle with and without enucleation of the corpus luteum. Injection of PMS during the luteal or follicular phase of the estrous cycle did not bring about any significant differences in treatment cycle lengths or in the ovulatory response; however, corpus luteum enucleation tended to hasten the onset of estrus. The mean ovulation rate for the heifers receiving the day 16 injection was 5.9 for the intacts and 6.0 for the heifers undergoing corpus luteum enucleation, respectively. The number of heifers returning to estrus suggested that the enucleation caused a detrimental effect on conception. They concluded that apparently there was no advantage in initiating treatment earlier than the 16th day of the cycle or by expressing the corpus luteum.

Schilling and Holm (1963), realizing that up to that time only about 30 percent of the treated cows ovulated the limited number of eggs (two or three) likely to lead to the birth of twins or triplets, conducted several small investigations trying to find a reliable sequence of hormone injections. Their first attempts ended in failure; however, promising results were obtained by the following treatment. On the fifth day after heat, eleven cows received a single dose of 1000 to 1500 I.U. PMS. The corpora lutea were enucleated between the 16th and 18th day followed by an injection of 2000 I.U. of PMS. After 3-5 days all the animals came into estrus and were then given 4000 I.U. LH intravenously. At slaughter two of the 11 cows failed to ovulate, one produced only one egg, four ovulated two eggs and four ovulated three eggs. Almost all of the ova were fertilized, the ovaries contained few surplus follicles and estrus was found to be normal. If the first PMS injection was omitted, fewer animals responded to treatment and numerous follicles were found on the ovaries. Their justification or basis for this treatment sequence was explained by the following argument. It was assumed that a ripe follicle begins to develop during or just after the last estrus and also it was believed that this follicle grows with others which become atretic. Their aim was therefore to prevent the process of atresia, or to stimulate the growth of new follicles by an additional dose of PMS given during the post-estrus period at day five.

Using this injection sequence, Turman et al. (1968) treated 81 beef cows with two subcutaneous injections of PMS: 1500 I.U. on day 4, 5, or 6 and 2000 I.U. on day 16, 17 or 18 of the cycle. On the day of estrus a dose of 2500 I.U. HCG was injected intravenously. The cows were hand mated and then pasture exposed for the remaining 2 to 3 months of the breeding season. Conception rate at the first post-PMS estrus was 64.2 percent, and over the entire breeding season 87.7 percent. Of 48 cows that conceived at the first post-PMS estrus, 25 produced singles and 23 produced multiple births (12 twins, eight triplets, two quadruplets and one quintuplet). Live calves included: 24 singles, 24 twins, 12 triplets, three quadruplets and two quintuplets. Multiple births were found to occur in 46.7%, 46.7% and 53.8% of the cows injected first on day 4, 5 or 6, respectively, and in 55.5%, 52.9% and 30.8% of those injected on day 16, 17 or 18 of the estrous cycle, respectively (estrus is day 0).

With superovulation work in the cow, Dziuk _et al_. (1958) obtained a much more consistent response in the number of follicles developing when he administered a relatively purified pituitary FSH extract of porcine origin than with PMS. Due to the wide variation in stimulatory response obtained with PMS and the present increased purification of gonadotropins from various species, FSH is beginning to be used more for the induction of twin pregnancy in cattle. Lotti and Galli (1961) injected 12 cows with 500 I.U. of FSH 12 to 14 days following estrus and then with 1000 I.U. 3 to 4 days later. Five cows produced singles, three produced normal twins and the other four aborted triplets or quadruplets. When 1000 I.U. of FSH was given as a single injection on day 17 or 18, eight cows produced normal twins, three produced normal triplets and two aborted (one set of twins and one set of quadruplets).

Dose-Response Relationship with Estrous Synchronization. An increasing amount of work is being directed towards the synchronization of estrous cycles in cattle by normal therapy. The use of progestogens for synchronization purposes has been reviewed by

Hansel (1967). The importance of estrus synchronization for broadening the acceptance of artificial insemination in beef cattle cannot be overestimated. The induction of twin pregnancy is likewise thought to have the greatest potential in cattle raised for beef purposes; therefore, it becomes almost essential that the gonadotropin treatments be combined with the synchronization treatment in order for practical application to ensue. Even though more unknown variables are brought into experimentation when a gonadotropin treatment is superimposed on a synchronization trial, several studies of this nature have been reported in the literature.

Nellor and Cole (1956) used various doses of PMS after ovulation was inhibited for a period of time with progesterone. Following the use of 2000 I.U. of a purified PMS preparation, ovulations varied from 1 to 15 in Hereford heifers. The multiple ovulations obtained were considered excessive rather than facillitory; therefore, a lower dosage of 750 I.U. of PMS was used on 20 heifers that were not all sexually mature. Of the 15 heifers showing estrus, three were pregnant at 60 days post-insemination with one normal set of twins and two normal singles. The use of progesterone followed by a single injection of equine gonadotropin resulted in a greater number of treated heifers coming in estrus than with progesterone alone. All of the heifers examined following this controlled estrus had ovulated; however, the conception rate following the controlled estrus was only 17 percent. Nellor _et al_. (1960) again using beef heifers, employed orally active progesterone compounds to control estrus and ovulation, but the injection of 1500 I.U. of PMS on the day following the cessation of progestational treatment did not result in any multiple ovulations. Similar results were reported by Lamond and O'Brien (1960) using beef cattle and daily injections of progesterone rather than oral administration. Ray et al. (1961) using 11 beef heifers, gave a single subcutaneous injection of 560 mg. crystalline progesterone in a starch suspension at various stages of the estrous cycle followed by a single subcutaneous injection of 750 to 2250 I.U. of PMS. The interval from PMS injection to estrus was 2 to 38 days with no detectible grouping in the return to heat. In contrast, Nellor and Cole (1956) reported that 79 percent of the heifers receiving a similar treatment were in estrus 0-4 days following PMS injection.

Jainudeen and Hafez (1966) fed 48 heifers at various stages of the estrous cycle 180 mg. 6-methyl-17-acetoxyprogesterone (MAP) daily for 18 days followed by 1000, 1500 or 3000 I.U. PMS given 24 hours after the end of MAP feeding and 1000 I.U. HCG immediately after the onset of estrus. Over 90 percent of the animals displayed estrus 2 to 6 days after cessation of MAP feeding. Mean ovulation rate of the females given 1000, 1500 and 3000 I.U. PMS was 2.8, 4.4 and 6.2, respectively. Conception rate at the synchronized estrus was 14 percent versus 57 percent for the heifers given 1500 I.U. PMS plus 1000 I.U. HCG but no MAP.

Bellows et al. (1969) used 77 beef heifers to determine FSH treatments that would give a controlled increase in the number of potentially fertile ova following estrus synchronization. Estrous cycles were synchronized by feeding 180 mg. MAP for either 9 or 11 days. On the second day of feeding, regression of the corpus luteum was induced by an intramuscular injection of 5 mg. estradiol valerate as outlined by Wiltbank and Kasson (1968). Porcine FSH (Armour) treatment levels were total dosages amounting to 3.12, 6.25, 12.50, 25.00, 50.00 or 75.00 mg. All heifers were laparotomized and bilaterally ovariectomized 60 to 72 hours following breeding. Total FSH dosages of 12.50 mg. or higher gave excessive stimulation. Average number of ovulations and fertilization rates were 1.12 and 92.9%, 2.12 and 93.8% and 8.00 and 79.4% for total dosages of 3.12, 6.25, and 12.50 mg. FSH, respectively. The most predictable ovarian response was obtained when FSH was injected twice daily on days 8, 9, 10, 11 and 12 of the treatment cycle.

The high proportion of double ovulations obtained in the above work with the 6.25 mg. FSH treatment sequence was the basis for a study done by Vincent and Mills (1969) with lactating crossbred cows. Eighty-four head were allotted to seven treatment groups. Instead of using a feeding regime with an orally active progestogen, a 5 mg. injection of norethandrolone was given intramuscularly daily for 4 days to inhibit estrus while FSH was given either once or twice daily for 5 days beginning with the first injection of norethandrolone. Injections were begun 14 to 16 days after the previous individual estrus. The ovulatory response as determined by rectal palpation found that 55 percent of all FSH treated cows had multiple ovulations and only four head (6%) had more than three corpora lutea. Injections made twice daily resulted in higher ovulation rates and more cows with multiple ovulations than did injections given only once daily. The conception rate for the FSH treated cows was 53% versus 93% for the controls. A second trial was performed where norethandrolone plus 7.8 mg. FSH were given twice daily as in the previous trial. This treatment group had a mean ovulation rate of 1.8 and a conception rate of 64% which was similar to that of the controls (57%) in this trial.

Development of Twin Fetuses. With advancing pregnancy, the embryo becomes increasingly dependent upon the placenta for its survival. The degree of placental development is primarily influenced by the availability of space and vascular supply within the uterus. As the number of implantations rise, the vascular supply to each site is reduced; this restricts placental development and causes high embryonic and fetal death (Eckstein *et al.*, 1955; McLaren and Michie, 1960; Adams, 1962; Hafez, 1964a). Erdheim (1942) observed 17 twin pregnancies in dairy cows and found that fifteen were bilateral and two were unilateral; in the pregnant uteri of 19 beef cows, he found sixteen were bilateral and three were unilateral. At six weeks of pregnancy Gordon *et al.* (1962) found 20 cows bearing twins unilaterally and 39 cows bearing twins bilaterally by rectal palpation. He reported fetal losses for these twin bearing cows to be 45 percent for the unilaterals and 31 percent for the bilaterals.

Hafez (1964b) studied the distribution of embryos after PMS and HCG injections were given to 42 nulliparous beef cattle. He observed that when the uterine horn contained one embryo, it occupied the middle or upper two thirds of the horn. With an increase in the number of embryos, the sites of attachment were unevenly spaced and overcrowded, causing a high degree of fetal mortality especially when the uterine horn contained more than two embryos. Gordon et al. (1962) observed that where three to six ova were shed by one ovary, a 57 percent survival rate of twins in cattle was observed in the corresponding uterine horn, but twins rarely survived when an ovary shed as many as 10 to 15 ova. They also reported that cows shedding only two ova were particularly liable to have only one surviving fetus. Survival of both ova was considerably greater when one ovum was shed by each ovary rather than both from the same ovary. Transuterine migration, which occurs regularly in the pig (Dziuk et al., 1962), occurs to a much lesser extent with multi-ovulating cattle and almost rarely does it occur with single ovulating cattle (Hafez, 1964b).

Ninety to 95 percent of the bovine twins are monochorial; i.e., the blood vessels of the two allantochorion membranes anastomose resulting in a constant interchange of blood between the two fetuses. This condition leads to the unique phenomenon of bovine freemartinism in fraternal twins where a heifer is born co-twin with a bull calf (Cole, 1916; Lillie, 1922). The freemartin heifer is sterile and its common occurrence has been the basis for argument against twinning in cattle by some investigators; however, the meat producing qualities of freemartins are not inferior to those of normal calves.

METHODS OF PROCEDURE

This experiment was conducted from June to December, 1968 at the beef facilities of South Dakota State University, Brookings, South Dakota. Thirty-six yearling Angus heifers weighing an average of 640 pounds and 36 yearling Hereford heifers averaging 630 pounds at the time of hormonal injections were used in this experiment.

The heifers were maintained under dry lot conditions and were fed a ration considered adequate for normal growth and reproduction. The heifers were observed for estrus twice and often three times daily. Any heifer which would stand to be mounted by another heifer was considered to be in estrus. All animals exhibited one normal estrous cycle (17 to 23 days) prior to treatment. The heifers were also palpated rectally before treatment for the detection of any gross abnormalities of the reproductive tracts.

Heifers were randomly assigned to four groups on the basis of individual body weight and breed (nine Angus and nine Hereford to each lot). For the obvious advantages of having the estrous cycles grouped for the subsequent injections to stimulate follicular development, a synchronization treatment was employed. Due to the lowered fertility presently known to occur at the first estrus following progestogen withdrawal, the different hormonal sequences were not administered until the estrous cycle following the first synchronized estrus. The heifers were fed a ration of 12 pounds of alfalfa hay, 4 pounds of rolled corn and 1 pound of protein supplement in order to get the heifers in a gaining condition prior to treatment. During the synchronization treatment, the one pound of commercial supplement contained 0.4 mg. melengestrol acetate (MGA)[1] per pound and was fed to all heifers for a period of 18 days.

Treatments

The estrus following withdrawal of the MGA from the feed was designated as day 0 of the subsequent estrous cycle. All injections were made on a particular day of the cycle with respect to this reference point. All treatments used in this experiment are presented in table 1.

All animals in this experiment were inseminated with frozen semen[2] that was collected from a single ejaculate of a fertile Angus and Hereford bull. The heifers were serviced once at what was considered the most optimum time for normal conception to occur (12-18 hours after the onset of estrus). Any heifer that continued to stay in estrus was given additional ampules as long as she remained in heat. The heifers were turned in with a fertile bull 30 to 35 days following insemination at the treatment estrus, and the bull remained with the heifers until they were slaughtered.

Ovarian Response

To accurately determine the amount of follicular stimulation and ovulation, a high lumbar laparotomy was performed on all heifers 8 to 12 days after artificial insemination. The operation was performed with the animal in the standing position similar to the technique of

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[1] 6-dehydro-16-methylene-6-methyl-17a-acetoxyprogesterone. Product of the Upjohn Co., Kalamazoo, Michigan.

[2] Gift of Curtiss Breeding Service, Cary, Illinois.

TABLE 1. EXPERIMENTAL DESIGN AND TREATMENTS USED IN THE EXPERIMENT

		Induced Multiple Ovulation Treatment[a]			Ovulation Induction Treatment[b]		
Treatment group	No. of animals	Day of injection	Dosage	Hormone	Day of injection[c]	Dosage	Hormone
I Controls	9 Angus	16	20 ml.	physiological saline		---	
	9 Hereford		20 ml.	physiological saline		---	
II	9 Angus	5	1500 I.U.	PMS[d]	21	1000 I.U.	HCG[f]
	9 Hereford	16	2000 I.U.	PMS[d]			
III	9 Angus	16	1500 I.U.	PMS[d]	21	1000 I.U.	HCG[f]
	9 Hereford						
IV	9 Angus	5	2.5 mg.	FSH[e]	21	1000 I.U.	LH[g]
	9 Hereford	16	3.0 mg.	FSH[e]			

[a]Injected subcutaneously.
[b]Injected intravenously.
[c]Injected at first sign of estrus or on the morning of day 21.
[d]Gonadin, Cutter Laboratories, Berkeley, California.
[e]NIH-FSH-S6 (ovine), National Institutes of Health (NIAMD).
[f]Jensen Salsbery Laboratories, Kansas City, Missouri.
[g]NIH-LH-B6 (bovine), National Institutes of Health (NIAMD).

Casida (1960). A local anesthetic[1] was used to prepare the heifers for surgery. Each heifer received a combiotic[2] injection at the time of the operation and the following day to keep the occurrence of any infection at a minimum. Notes were taken on estimated size of ovary, number, size and location of both follicles and corpora lutea.

All heifers were slaughtered 90-120 days after insemination at the treatment estrus. The reproductive tract of each heifer was recovered and data concerning the fetuses and ovaries were tabulated.

[1]5% hexylcaine hydrochloride, Cyclaine, Merk & Co., Inc., Rahway, New Jersey.

[2]Chas. Pfizer & Co., Inc., New York, New York.

RESULTS AND DISCUSSION

Due to the excessive amount of ovarian stimulation obtained in treatment groups II and III, it is obvious that the treatments exerted a significant effect upon ovulation rate. Results have been expressed in percentage response or as means with ranges. The main emphasis has been placed on comparisons within and between treatment groups II and III because of the more equal number of heifers being multiply stimulated using the same gonadotropin.

Ovarian Response

Follicular Stimulation and Ovulatory Response. In an attempt to accurately determine the total amount of ovarian stimulation in each heifer, the number of follicles reaching an estimated size of 10 mm. in diameter or greater as well as the number of ovulations was noted at the time of laparotomy. McNutt (1924) reported that most follicles in normal cycling cattle reach a diameter of 16-19 mm. just prior to rupture; however, the size reached is very irregular and some follicles will ovulate at the time that they are only 10 mm. in diameter. Ulberg *et al*. (1951) found the diameter of 43 follicles in non-treated cattle at the first sign of estrus to range from 10 to 18 mm. with an average of 14.1 mm.

The total ovarian response for each treatment group is presented in table 2. The amount of stimulation in groups II and III when considering both the number of follicles $>$10 mm. and the number of ovulations was found to be quite variable. The heifers in treatment II which received two injections of PMS totaling 3500 I.U. had a

TABLE 2. TOTAL OVARIAN RESPONSE FOR EACH TREATMENT GROUP

Treatment group	Number of heifers stimulated[a]	Number of follicles >10 mm.[b]		Number of heifers ovulating	Number Ovulations		
		Range	Mean		Range	Mean	As % of follicles >10 mm.
I	1[c]	1-3	2.00	18	1-2	1.05	52.7
II	18	4-38	25.50	16	1-16	7.37[d]	31.9
III	16	2-39	13.60	16	1-13	5.31[d]	34.7
IV	6	1-7	2.55	18	1-6	1.39	54.3

[a]Designated as those heifers where at least two ovulations or two follicles > 10 mm. were present at laparotomy.

[b]Includes the number of ovulations.

[c]One heifer spontaneously ovulated twice.

[d]Mean figured only heifers actually ovulating.

stimulatory response in the number of follicles $>$10 mm. almost double to that found in the treatment III group receiving a single PMS dosage of 1500 I.U. At the greater PMS dosage level, multiple stimulation was observed in all 18 heifers, whereas with the lower PMS level, two heifers did not respond to treatment. No difference was found in the maximum individual number of follicles $>$10 mm. produced within each PMS treatment group.

The mean ovulation rate for treatment II was 7.37 as compared to 5.31 for treatment III. Even though treatment II produced a greater number of follicles and a higher ovulation rate, the total number of ovulations when based as a percentage of the total number of follicles $>$10 mm. was found to be no different from that for treatment III. Two heifers failed to ovulate in each PMS group. In group II one heifer had 33 and the other had 32 follicles $>$10 mm. Twenty-five follicles were found in one heifer and five were found in the other heifer in treatment III which failed to ovulate.

Eighty-four percent of the heifers in group II responded to treatment with two or more ovulations as compared to 73 percent for treatment III (table 3). With only six heifers from treatment II and seven heifers from treatment III ovulating between two and six eggs, which is the desirable range for the production of multiple births, it is questionable whether or not the additional injection of PMS on day 5 of the cycle (treatment II) is beneficial for the induction of a limited number of ovulations. Half of the heifers in treatment II were found at laparotomy to have seven or more corpora lutea (C.L.)

TABLE 3. PERCENTAGE OF HEIFERS WITH MULTIPLE OVULATIONS

Treatment group	Number of Ovulation Sites										Number of heifers with multiple ovulations	% of heifers with multiple ovulations
	0	1	2	3-4	5-6	7-8	9-10	11-12	13-14	15-16		
I	-	17 (94%)	1[a] (6%)	-	-	-	-	-	-	-	1 (18)	6%
II	2 (12%)	1 (6%)	2 (12%)	3 (16%)	1 (6%)	3 (16%)	1 (6%)	3 (16%)	1 (6%)	1 (6%)	15 (18)	84%
III	2 (12%)	3 (16%)	4 (22%)	-	3 (16%)	2 (12%)	2 (12%)	1 (6%)	1 (6%)	-	13 (18)	73%
IV	-	15 (82%)	2 (12%)	-	1 (6%)	-	-	-	-	-	3 (18)	16%

[a]One heifer spontaneously ovulated twice.

compared to one-third of the 18 heifers in treatment III. The highly variable individual response to injections of PMS which was observed in this study has been reported by other workers (Gordon et al., 1962; Hafez et al., 1964, 1965; Jainudeen et al., 1966; Scanlon et al., 1968).

The calving response obtained by Turman et al. (1968) with cows ranging in age from 2 to 5 years seems highly desirable. However, no attempt was made by these researchers to determine the ovulation rate. Treatment II in the present study employed the same PMS dosage levels and the most desirable injection sequence (day 5 and day 16) used by the Oklahoma workers. In contrast to the results obtained by Turman et al. (1968), the results of the present study indicate that the level of PMS used in treatment II causes excessive ovarian stimulation of nulliparous heifers.

With a limited amount of purified ovine FSH and no standard dosage level to use as a base, treatment IV was broken up into two injections. It was hoped that the small dosage levels of FSH given on day 5 and day 16 might produce some information as to the possible importance of a day 5 injection. Thirty-three percent of the heifers responded to treatment; however, only three (16%) were found possessing multiple ovulations. Two heifers produced twin ovulations, one heifer had six ovulation sites and the rest of the heifers were found with single ovulations. Recent work by Bellows et al. (1969) and Vincent and Mills (1969) shows a fairly predictable response with 6.25 mg. of FSH in producing a low level of multiple ovulations. Their response was more predictable with twice daily injections given for a period of 5 days than for other injection sequences. The time and labor that would be involved with several injections of this nature when considering several animals would not provide for a practical or economical outcome. The numerous injections are believed to be necessary due to the rapid degradation of the protein structure possessed by FSH and the other pituitary gonadotropins. Bellows et al. (1969) reported that FSH injected twice daily resulted in a marked reduction in the amount required to produce ovarian stimulation. This is probably due to the maintenance of a more constant hormone level in the blood.

PMS on the other hand is generally found to be relatively long acting and the division of a standard dosage into two or three smaller injections does not aid the ovarian response. Hafez et al. (1963a) found a single injection of PMS to be superior in ovulatory response to two or three injections of the same total dosage. The fact that the total dosage of FSH used in treatment IV was only 5.5 mg. and that one-third of the heifers were stimulated by the day 5 and day 16 injection sequence may merit further study.

The time interval between the day 16 injection of PMS or FSH and estrus has a pronounced effect on the ovulation rate (table 4). The trend is for a greater percentage of follicles developed to ovulate as the interval from the gonadotropin injection to estrus increases. From this study an interval of at least four days seems to be necessary for a respectable number of ovulations to occur. The day 5 interval accounted for 50.6 percent of the follicles $>$10 mm. ovulating. A similar relationship has been reported previously by Rowson (1951), Brock and Rowson (1952), Hafez et al. (1963a) and Scanlon et al.

TABLE 4. PERCENTAGE OF FOLLICLES OVULATING IN RELATION TO THE INTERVAL BETWEEN PMS OR FSH INJECTION[a] AND ESTRUS IN THE STIMULATED HEIFERS[b]

	Interval (days) PMS or FSH to Estrus							
	1	2	3	4	5	6	>6	All intervals
Number of heifers	1	4	4	5	6	4	2	26
Number of follicles >10 mm.[c]	34	50	103	93	85	77	30	472
Mean No. of follicles	34.0	12.5	25.6	18.6	14.2	19.2	15.0	
Number of ovulations	0	8	8	31	43	19	19	128
Mean No. of ovulations	00.0	2.00	2.00	6.20	7.17	4.75	9.50	
Ovulations as a % of the follicles >10 mm.	0.00	16.0	7.76	33.3	50.6	24.6	63.3	36.9

[a]Injected on day 16 of estrous cycle.
[b]Those heifers with at least two ovulations or two follicles >10 mm. at laparotomy; eleven heifers each from groups II and III and four from group IV.
[c]Includes the number of ovulations.

(1968). The reason for the low ovulatory response in the four animals at the day 6 interval cannot be explained.

This increased ovulatory response has been noted by Gordon et al. (1962) to occur until an interval of 8 days has been reached after which it declines rapidly. Two heifers from treatment group II had an interval of greater than 6 days. An interval of seven days in one heifer allowed 70 percent (5 of 7) of the follicles $>$10 mm. to ovulate. A heifer with an eight day interval was found to have 23 follicles $>$10 mm. and 14 ovulation sites for a percentage of 60 percent. There does not seem to be any trend in the data to suggest that the number of follicles $>$ 10 mm. increases with the interval from injection to estrus.

The greater ovulatory response obtained on day 5 would seem to be due to the fact that these animals are showing estrus at a normal cycle interval and that the hormonal factors associated with such regularity are optimum for allowing response to the administration of an exogenous gonadotropin. It is assumed that if this 5 day interval does not occur, the maturity of many of these follicles is probably such that they are not capable of ovulating normally.

The injection to estrus interval is affected by two variables: (1) the time at which PMS or FSH is administered after the previous estrus and (2) the individual variability in estrous cycle length. The first variable can be alleviated by making a single PMS injection on approximately day 16 of the estrous cycle, which is presently considered the most desirable. The second variable, however, seems very difficult to control as evidenced by the very low repeatability (0.069), that Olds and Seath (1951) found for estrous cycle length. Because of this low repeatability, it would not be possible to estimate reliably the most desirable injection date (allowing for a 5 day interval) for a given cow even if previous cycle length data is available.

Individual body weight was hoped to explain some of the variation found in the stimulatory response (table 5). With a limited number of heifers of light weight no trend could be found to support the theory that heifers of light body weight would show a greater ovarian response to a given treatment. Gordon et al. (1962) found evidence that smaller animals (below 900 lbs.) responded to treatment more readily than larger animals (over 1100 lbs.). Further evidence for ovulation rate was in favor for the smaller cows. Bellows et al. (1969) found that the correlation between body weight and the number of ovulations was small and nonsignificant in a group of heifers ranging from 850-1200 pounds.

All heifers in this study were approximately of the same age. Gordon et al. (1962) found that the age of the cows is not of major importance in determining whether or not the animal will shed additional eggs. The literature on the natural incidence of twinning often records twinning incidence to be related to age. It has been suggested that it is not certain whether this is due to a rising incidence of multiple ovulations with increasing age or that multiple pregnancy is more readily sustained by older cattle.

Past studies, mostly with dairy cattle, have reported significant differences between breeds of cattle and the incidence of twins. The possibility that some variability in response to exogenous gonadotropin might arise between the two breeds in this experiment found

TABLE 5. OVARIAN RESPONSE IN RELATION TO INDIVIDUAL BODY WEIGHT[a]

Weight ranges	Number of heifers	Number of follicles > 10 mm.[b]	Average number of follicles > 10 mm.[b]	Number of ovulations	Average ovulation rate	Ovulations as a % of follicles > 10 mm.
500-550	3	60	20.0	18	6.00	30.0
550-600	8	118	14.7	37	4.62	31.3
600-650	14	212	15.1	92	6.57	43.4
650-700	9	205	22.8	49	5.44	23.9
700-750	2	36	18.0	5	2.50	13.9

[a]Weights from the heifers in treatment groups II and III were used to prepare this table.
[b]Includes the number of ovulations.

no basis for support of either breed (table 6). Gordon et al. (1962) with six pure breeds and four breed crosses likewise was unable to find any clear indication of a particular breed or cross that showed a greater number responding to treatment or that showed a greater ovulatory response. Turman et al. (1968) reported that 11 of 15 crossbred cows produced multiple births in comparison to 12 of 33 straightbred mature Hereford and Angus cows.

It has been shown several times that ovulation is distributed at random between the ovaries of several polytocous animals, but there is much evidence to show that this is not true for the cow and the ewe. In the cow the evidence appears to be overwhelming in support of the right ovary as being the most active. Kidder et al. (1952) reported that there is no systematic sequence of ovulations from one ovary or the other. The right uterine horn has been reported consistently to be pregnant more often than the left (Perkins et al., 1954). Erdheim (1942) reported a 2:1 ratio of pregnancies favoring the right horn of the uterus in 1506 dairy animals, but almost equal distribution was found in 2318 beef animals. Gordon et al. (1962), checking on the number of corpora lutea induced after PMS treatment, found 59.1 percent of the ovulations occurring in the right ovary and 40.9 percent in the left. In the present study 54.2 percent of the total number of follicles >10 mm. occurred in the right ovary (table 7). The total number of ovulations for all groups also showed some favor to the right ovary (53.0%).

Effect of the Estrous Cycle on Ovarian Response. When comparing the cycle lengths of the three groups treated with a gonadotropin to

TABLE 6. COMPARISON OF OVARIAN RESPONSE BETWEEN BREEDS

Treatment group	Breed[a]	Number of follicles[b] > 10 mm.	Number of ovulations	Ovulations as a % of follicles > 10 mm.
I	Angus	19	10	52.6
	Hereford	17	9	52.9
II	Angus	185	64	34.9
	Hereford	186	54	29.0
III	Angus	131	37	28.3
	Hereford	114	48	42.1
IV	Angus	23	11	47.8
	Hereford	23	14	60.9

[a]Nine Hereford and nine Angus in each treatment group.
[b]Includes the number of ovulations.

TABLE 7. COMPARISON OF OVARIAN RESPONSE BETWEEN LEFT AND RIGHT OVARIES

Treatment group	Number of Follicles > 10 mm.[a]		Number of Ovulations	
	Left ovary	Right ovary	Left ovary	Right ovary
I	20	16	10	9
II	165	205	56	62
III	115	130	41	44
IV	19	27	9	16
Total	319 (45.8%)	378 (54.2%)	116 (47.0%)	131 (53.0%)

[a]Includes the number of ovulations

those of the controls, there is little evidence to suggest that treatment affected their length (table 8). The mode for these three groups was 20 days. In sheep there is general agreement that when PMS is given in the follicular phase of the cycle the onset of the next estrus is hastened somewhat (Robinson, 1951; Wallace, 1954; Gordon, 1958); however, there is no evidence for this in the cow. The group II heifers receiving a total dosage of 3500 I.U. of PMS had a cycle interval similar to that of the controls and those receiving a considerably lower dosage level of a gonadotropin.

The use of PMS or FSH has been reported to cause a high incidence of silent estrus whereby the cow ovulates as she would under normal conditions, but the external signs of receptivity to the bull or other cattle attempting to mount her are not exhibited. Dziuk et al. (1958) noted that when FSH was given near the time of the expected estrus, several cows did not exhibit normal symptoms of estrus. Gordon et al. (1962) reported the incidence of silent heat in cows given PMS injections to be 14 percent.

Silent estrus was observed in all four treatment groups (table 8). This phenomenon occurred in 21 percent of all heifers. There appears to be a basis for assuming that its incidence occurs more often with PMS treated cattle, as groups II and III each contain five animals in which estrus was not observed. However, its occurrence is known to be common in untreated cattle, and Hafez et al. (1965) reported that no significant difference was observed in its incidence in cattle treated with PMS and those that were not. Kidder et al. (1952), Trimberger and Fincher (1956) and Labhsetwar et al. (1963b) have

TABLE 8. COMPARISON OF OVARIAN RESPONSE IN HEIFERS SHOWING ESTRUS TO THOSE HAVING A SILENT ESTROUS PERIOD

	Treatment Groups			
	I	II	III	IV
Heifers Observed in Estrus:				
Number	15	11[a]	13	16
Treatment estrous cycle				
Range (days)	17-29	17-24	18-22	18-27
Mean	20.9	20.8	20.0	20.0
Ovarian response				
Mean No. of follicles >10 mm.[b]	2.00	23.5	15.4	2.37
Mean No. of ovulations	1.06	7.33(9)[c]	5.33(11)[c]	1.37
% ovulations of follicles >10 mm.	53.3	25.5	31.8	57.8
Heifers Having a Silent Estrous Period:				
Number	3	5	5	2
Ovarian response				
Mean No. of follicles >10 mm.[b]	2.00	10.4	8.80	4.00
Mean No. of ovulations	1.00	6.40	5.22(4)[c]	1.00
% ovulations of follicles >10 mm.	50.0	61.5	47.7	25.0

[a]Two heifers are not included: One heifer showed estrus one day prior to the day 16 injection, another heifer showed estrus three days following the day 5 injection.
[b]Includes the number of ovulations.
[c]Number of heifers ovulating.

reported the percentage of quiet ovulations to range from 18 to 27 percent of all estrous cycles in cattle. It is possible that estrous periods of a very short duration could go undetected as suggested by Hall *et al.* (1959).

A comparison of the ovarian response of the heifers showing estrus to those believed to have a silent heat is presented in table 8. It is interesting to note that in both PMS groups the mean number of follicles $>$10 mm. for those heifers not observed in estrus is significantly less than that reported for the heifers showing estrus. However, the percentage of the follicles $>$10 mm. which ovulated is considerably higher in the heifers not exhibiting estrus than in those showing estrus. Scanlon *et al.* (1968) did not observe estrus in 10 of 89 head of cattle that were subjected to high PMS dosage levels. The mean ovulation rate of those not showing estrus was 18.9 which was markedly above that for all estrous animals (9.0) and also above the mean (12.7) for those in estrus at the 5 day interval after PMS treatment. In contrast Avery *et al.* (1962) reported that cows exhibiting estrus have a higher superovulation response than those not showing estrus.

From the observation of Trimberger and Fincher (1956) and from the few animals that were found pregnant at slaughter in this study, the conception rate in artificially inseminated animals not showing estrus is believed to be satisfactory; however, the most optimum time to breed presents a problem. The precise control of ovulation time obtained by Graves and Dziuk (1968) after cattle had undergone a synchronization treatment indicates that large groups of cows may in the future be successfully inseminated at some predetermined time without even detecting estrus. Hafez et al. (1963b) injected estrogen after PMS treatment and found that the ovulation times were more compact and occurred in response to administered HCG more readily.

The use of MGA to synchronize the estrous cycles of the heifers in this experiment prior to the cycle where the hormonal injections were made was not totally successful. After the withdrawal of the oral progestagen, 68 of the 72 heifers showed estrus within 5 days. The problem was that the second post-treatment estrous cycle length was found to be quite variable. A few heifers came back into estrus within 10 days after the synchronization estrus. It was felt that the 0.4 mg. level of MGA was evidently not adequate to successfully control ovulation. Bellows (1969) obtained similar results with 1.0 mg. of MGA fed for 11 days (treatment scheme upon which F.D.A. approval of the hormone for synchronization purposes has been requested). They found that the estrous cycle following synchronization estrus varied from 5-31 days.

Split estrus -- estrus interrupted by one or more days of non-receptivity to the bull -- was the chief abnormality encountered after PMS injection by Hafez et al. (1965). The frequency of split estrus was inversely related to the time interval between the PMS injection and the subsequent estrus. The occurrence of this condition in the trial was observed in three heifers. From the insemination records these three heifers were inseminated at the proper time and then inseminated again after an interval of approximately one to three days. Two of these heifers were found in estrus 2 days after gonadotropin treatments.

The artificial insemination dates and the number of ampules used per heifer suggest that the PMS treated heifers were in estrus longer than the controls or the FSH treated cattle. The cattle were given additional ampules as long as they remained in estrus. The number of ampules used per heifer in groups II and III averaged 2.0 compared to 1.4 ampules for groups I and IV. This could possibly be explained by the fact that some of these heifers were stimulated to a fairly high level and that the long estrous periods were due to the estrogen production by the numerous follicles prior to ovulation, which could be occurring over this period. There appears to be a trend in the data to the effect that a greater number of ampules were used on heifers having excessive amounts of follicular development; furthermore, the number of ampules used per heifer tended to be lower with those having a high percentage of ovulations.

In the PMS heifers a greater amount of difficulty was observed in the successful completion of the insemination technique. For groups I, II and III the number of heifers which provided difficulty in passing the insemination pipette through the cervix were three, six and seven, respectively. No heifer in group IV provided any insemination problem; the possible meaning of this observation cannot be explained.

Fertility Following Treatment

Heifers Pregnant to Treatment at Slaughter. A very low percentage of those cattle treated with PMS was found to be pregnant at the time of slaughter (table 9). Only 12 percent (2) of the heifers receiving the total PMS dosage of 3500 I.U. were pregnant, while 24 percent of the heifers receiving 1500 I.U. of PMS settled to treatment. The conception rate was considerably higher in the control heifers (61%) and the heifers receiving 5.5 mg. of FSH (44%). However, only six heifers receiving FSH were multiply stimulated and only three ovulated more than one ovum.

The fertilization rates of cattle receiving gonadotropic hormones are reported in the literature as estimates based on the cleavage stages of eggs recovered approximately 2 days after the last ovulation. Scanlon et al. (1968) found an average of 4.4 cleaved eggs in 79 superovulated cattle showing estrus and being bred by a fertile bull. This represented about 50 percent of the eggs shed and 63 percent of those eggs recovered. For the 10 heifers that were not observed in estrus and were artificially inseminated at day 21, a very marked reduction was found in the percentage of eggs undergoing cleavage. They also confirmed a finding by Hafez et al. (1963a) that fertilization tended to occur as readily in cattle shedding numerous eggs (more than 10) as it did in cattle ovulating only two or three eggs.

Scanlon et al. (1968), after comparing their data with that previously reported for the untreated ovulating cow (Hamilton and Laing, 1946), suggested that the superovulation treatment did not appreciably affect the cleavage stages reached by eggs in the early period following ovulation. The indications were that a majority of the eggs shed were capable of normal development. Bellows et al. (1969) found fertilization rate tended to be somewhat lower in cattle that were

TABLE 9. NUMBER OF HEIFERS PREGNANT AT SLAUGHTER

	Treatment Group			
	I	II	III	IV
No. of heifers pregnant to artificial insemination at estrus following treatment	11 (61%)	2 (12%)	4 (22%)	8 (44%)
No. of heifers pregnant to natural mating at third or subsequent estrus following treatment	4 (22%)	12 (66%)	8 (44%)	4 (22%)
No. of heifers remaining open at slaughter	3 (16%)	4 (22%)	6 (34%)	6 (34%)

treated with FSH for twin ovulations. Kidder _et al_. (1952) observed the conception rate in cattle producing single ovulations to be 58 percent as compared to only 29 percent for those that possessed multiple ovulations.

Hammond, Jr. and Bhattacharya (1944) reported that PMS does not alter the time relation between estrus and ovulation; therefore, faulty timing between insemination and ovulation does not appear to be involved as an explanation for the lowered fertility observed.

Turman _et al_. (1968) used a PMS injection sequence similar to that of group II and obtained, with the use of natural service, a conception rate at the first post-PMS estrus of 64.2 percent. Overall conception rate in the cows was 87.7 percent.

Gordon _et al_. (1962) found 317 of 416 cows (76.2%) artificially inseminated after PMS treatment to be pregnant 6 weeks after insemination. They further reported that the cattle conceived as readily after an injection of 2000 I.U. as they did after only an 800 I.U. dosage level. Where animals shed more than six eggs, the conception rate was found to be 84 percent.

It is possible that apparently unfertilized ova are actually fertilized ova undergoing early degeneration as a result of unfavorable hormone levels. Staples and Hansel (1961) have shown in the normal cow that a threshold of progesterone production represented by a luteal progesterone level of the order of 100 mcg. is necessary for embryo survival at the 15th day of pregnancy. It also is possible that some of the ova shed at the time of ovulation have not reached a proper stage of maturity and fertilization failure occurs or, if fertilization does take place, abnormal cleavage may ensue. As suggested by Dowling (1949) for the superovulated cow and by Robinson (1951) for the ewe, abnormal tubal transport in the form of acceleration may be responsible for the lowered fertility. Scanlon et al. (1968), however, have reported essentially similar results for superovulated heifers as well as for untreated animals.

Kidder et al. (1954) estimated that in a population of first service heifers, 60 percent would carry live embryos 60-90 days after insemination, 3 percent would not be pregnant due to abnormalities of the reproductive tract, 10 percent would shed defective ova, 12 percent would have fertilization failure and 16 percent would have embryonic mortality. It would probably be safe to assume that in multiply induced first service heifers the percentage of embryonic mortality would be increased.

Gordon et al. (1962) showed evidence that embryonic mortality occurs to a considerable extent in cows treated with PMS. Their data on the number of eggs surviving as fetuses in the 6th week following mating, in relation to the initial number of ovulations, clearly show a heavy loss in eggs in these early stages of pregnancy. Although 41.6 percent of the cattle shed additional eggs, only 23.1 percent were found to possess multiple fetuses at 6 weeks.

Embryonic mortality in cattle may be of two forms. In the first form, the ovum becomes fertilized, develops for a few days, but dies before midcycle. The corpus luteum then regresses as in a normal cycle when fertilization has not taken place, and the animal returns to estrus after one normal cycle length. In the second form, fertilization occurs and the ovum develops beyond midcycle before degeneration begins with resorption of the embryo; the regression of the corpus luteum is thus delayed until after a length of time longer than one estrous cycle.

The majority of embryonic deaths in single pregnancies occurs between the sixteenth and twenty-fifth day after insemination (Hawk et al., 1955). Hawk et al. (1963) reported that this embryonic death is more likely to be caused by factors other than the regression of the corpus luteum. This period of high embryonic mortality coincides with the rapid growth and differentiation, not only of the embryo proper, but also of the extra - embryonic membranes. Furthermore, the bovine embryo's survival is dependent upon its absorption of nutrients contained in the "uterine milk" from about the sixteenth day of gestation (Greenstein and Foley, 1958) until fetal-maternal union is established at about 36 days of pregnancy (Foley and Reece, 1953). The stage at which embryonic mortality occurs most often or exerts its effect on multiple pregnancy is not known and awaits further research. Hafez et al. (1965) reports a tendency for heifers not conceiving after PMS treatment to have a subsequent cycle interval somewhat greater than normal.

The heifers in groups I and IV that were found to be pregnant at slaughter all possessed single fetuses. One of the two heifers pregnant in group II had a double ovulation at the time of laparotomy and was found to have a single fetus and one corpus luteum at slaughter, while the other heifer was found to have all 12 corpora lutea present which were observed at laparotomy and five fetuses undergoing the process of absorption.

Four heifers were pregnant in treatment group III. One pregnancy resulted from a single ovulation in which no multiple follicular stimulation was observed. The left ovary of a second heifer was found to possess two corpora lutea at the time of laparotomy. At the time of slaughter twin fetuses were both found in the left uterine horn. At laparotomy four corpora lutea were found in the left ovary and one corpus luteum was found in the right ovary of a third heifer. However, at slaughter this heifer possessed only three corpora lutea, all on the left ovary. She also was found to be carrying a normal fetus in each uterine horn. A fourth heifer was found to contain a set of quadruplets undergoing absorption. The nine corpora lutea present on her ovaries at laparotomy were still present at slaughter. Only two heifers of the 54 heifers treated with hormones were carrying normally developing multiple fetuses at slaughter.

Heifers Pregnant to Natural Mating. To get some idea of the fertility of the heifers following gonadotropin treatment, a fertile bull was turned in with them 30-35 days after artificial insemination at treatment. The reason for the bull not being turned in immediately after treatment was to allow for an accurate determination of the number of animals pregnant to treatment at the time of slaughter when crown-rump measurements would have to be taken to determine fetal age.

The number of heifers that were found to have settled to the bull after the treatment cycle is shown in table 9. Approximately two-thirds of these cattle settled at the first estrous period after the bull was turned in. The rest settled at the second estrous period. The small number of cattle observed in estrus at the first cycle following treatment estrus and prior to turning the bull in suggests that the number of cattle returning to estrus, tend to do so in period greater than that of one normal estrous cycle.

At the time of slaughter (approximately 90-120 days from treatment estrus) 16 percent (3) of the heifers in the control group remained open as compared to 22 percent (4) for group II, 34 percent (6) for group III and 34 percent (6) for group IV. At slaughter the heifers remaining open in groups I and IV all appeared to be cycling normally. Three of the six heifers that were stimulated in the FSH group remained open. In treatment II two of the open heifers were found to be cycling, while the other two were both found to have retained a large corpus luteum containing a fluid filled cavity. Five of the six heifers unsettled in treatment group III were cycling. One had a large cystic follicle which was suspected prior to slaughter due to her continual estrous behavior.

GENERAL DISCUSSION

For those cattle showing estrus evidence suggests that the main factors controlling ovulation rate are the number of follicles capable of responding to a gonadotropin and the time interval elapsing between the gonadotropin injection and the onset of heat. Where the interval can be controlled to 5 days, a greater proportion of the mature follicles seem capable of ovulation. It further seems that the follicles of cattle which come into estrus and receive either HCG or LH in the early days after gonadotropin administration are not of sufficient maturity to ovulate in response to the exogenous LH.

The factors which determine the number of follicles that are responsive to PMS or FSH given at day 16, or for that matter at any time during the cycle, presently remain unclear. Where the desire is to produce a limited number of ova (two to six), the problem seems to be more complex, as evidenced by the lowered number of cows that respond to low levels of FSH or PMS with multiple ovulations. Even in those cows that receive a large PMS or FSH dosage and still do not show ovarian stimulation, it may be that something inherent prevents their development of additional follicles. Much work is still needed to determine the most desirable hormone preparation, dosage level and injection sequence.

The variability of response to a given dosage level of PMS noticed in this study and reported by numerous other research workers indicates a very important need to try and improve the reliability of this effective, cheap and readily available source of FSH. If this cannot be done, the use of other pituitary gonadotropins should be used at the research level where a fairly reliable stimulatory response is essential to the establishment of the most satisfactory dosage level and injection sequence for production of twin pregnancy.

Whether or not the follicle stimulating preparation used should be of a purified FSH origin or of an FSH-LH combination is not certain. LH is generally considered to be in greater supply than FSH in the bovine. It has been suggested by Varian et al. (1968) that the pituitary storage and secretion of LH may occur simultaneously. The release of LH activity during the mid-luteal phase of the cow has been indicated by increased follicular development (Rajakoski, 1960) and estrogen production (Hansel et al., 1949). It is felt by some researchers that the problem is to develop follicles in the ovary that are capable of responding to this endogenous LH. If this is the case, then small dosages of purified FSH should provide for most optimum development of two or three follicles.

The highly variable response obtained with PMS and some pituitary preparations may be due in part to the fact that they contain a fairly high LH component (Schmidt-Elmendorff et al., 1962a). This LH component is thought to possibly cause luteinization of the follicles prior to the time of their ovulation. The inactivation of this LH component in the various pituitary extracts and PMS with the use of six molar urea (Ellis, 1961; Schmidt-Ellmendorff et al., 1962b) would seem to merit study.

Human chorionic gonadotropin (HCG) is generally considered to be ovulatory and luteinizing in action. However, there is evidence that

HCG may also have follicle stimulating properties as well. In cattle with normal (Donald and Anderson, 1953) or with cystic ovaries (Hancock, 1948) an increased number of double ovulations occurs following HCG treatment. In sheep the ovulation rate after the injection of 1000 I.U. of HCG was comparable to that after the injection of 500 I.U. of PMS (Braden et al., 1960).

The most desirable dosage level should be considered as the one stimulating the greatest number of cattle to respond consistently with from two to six ovulations. This dosage level should furthermore either produce the two to six ovulations or should allow the cow to function as normal with a single ovulation. It is very important that dosage levels providing an excessive amount of stimulation (ten to twenty) be lowered due to the fact that twins are rarely sustained (Gordon et al., 1962). At the present time based on our results with PMS, a dosage level of 1500 I.U. can only be considered as causing an excessive amount of stimulation in nulliparous heifers.

The most successful treatment sequence as to the time of an injection or injections still requires further study. Ideally the minimal effective dose should be administered at the stage of the cycle when it will induce a high conception rate. Presently the day 16 injection seems to be the most desirable single injection sequence. As to the sequence of injections at day 5 and at day 16, the importance of the first injection on day 5 remains unclear. It may very well be beneficial especially at lower dosages; however, it may very well be of no importance and the response received may only be due to the day 16 injection. The data tend to show that the mean ovulation rate for this treatment sequence is comparable to that of other researchers using a 2000 I.U. PMS dosage level instead of a 3500 I.U. level.

The sensitivity of ovarian tissue to an exogenous source of PMS or FSH may explain some of the variability found from animal to animal. This could be affected by various factors such as: age, parity, stage of lactation, interval from last calving, plane of nutrition and season of year. The one particular factor that seems to require some study is parity. The comparison of the ovarian response between nulliparous heifers and cows to varying gonadotropin levels, taking into consideration body size, should provide information as to whether or not the ovarian tissue of heifers is more sensitive to stimulation. The excessive stimulation obtained with the heifers in this trial when compared to the results of other researchers using both heifers and cows tends to make one think that the ovarian tissue of the heifer is more sensitive. Maybe more important in the heifer is the problem imposed upon her to successfully carry multiple fetuses to term during her first pregnancy.

The maintenance of multiple pregnancy in the bovine appears to be very critical. The factors that affect it and their relative importance are not known. The higher the number of ovulations the lower is the ability of the animal to maintain pregnancy at 3 to 4 months of gestation. A bilateral twin pregnancy is much more likely to succeed to term than a unilateral one.

The physiological factors involved in the complete loss of whole litters are not fully understood. In polytocous species, increases in litter size reduce fetal growth because of the competition between individual fetuses. The retardation of fetal growth in individuals of large litters becomes much more marked as pregnancy proceeds. In monotocous species twin fetuses are generally smaller than single fetuses. The nutrition of the dam during the latter stages of pregnancy affects the growth of twin fetuses more than that of single ones (Wallace, 1948; Palsson and Verges, 1952; Hafez et al., 1968).

It may be possible that an unbalanced production of some gestagen is responsible for the inability of cows to maintain multiple pregnancies. The corpus luteum has been found to play an important role in maintaining pregnancy during the first three months of gestation and then its function declines somewhat thereafter (Stormshak and Erb, 1961). Although progesterone is necessary for the maintenance of pregnancy, the lowest concentration of progesterone is during late pregnancy (Gorski et al., 1958). The presence of progesterone in the bovine placenta has not been detected (Short, 1956). The large number of ovulations and the subsequent luteal development does not provide basis for an assumption that a progesterone deficiency is the cause of pregnancy failure. However, the progesterone requirements during multiple pregnancy are not known.

Repeated PMS or FSH injections have been shown by several researchers to be ineffective in inducing multiple ovulations. The nature of this ovarian refractoriness to repeated gonadotropin injections in cattle is not understood. Long periods of rest without the administration of gonadotropins do not seem to eliminate the refractoriness (Willet et al., 1953). The possibility that a homologous gonadotropin may not induce the antigonadotropin production remains to be investigated in the cow. At the present time there is no known source of a highly purified bovine FSH extract. Antigonadotropins to PMS and FSH exert their effect by limiting follicular development; however, the exact mode of action is still unknown.

SUMMARY

This study attempted to compared the ovarian response between two different injection sequences of pregnant mare serum (PMS) and one of FSH in 72 yearling Hereford and Angus heifers that were allotted to four treatment groups. Hormonal treatments to induce multiple ovulations were: I-controls, 20 ml. physiological saline on day 16 of the estrous cycle; II-1500 I.U. PMS on day 5 and 2000 I.U. PMS on day 16; III-1500 I.U. PMS on day 16; IV-2.5 mg. ovine FSH on day 5 and 3.0 mg. ovine FSH on day 16. After these subcutaneous injections, at the first sign of estrus or on the morning of day 21, groups II and III each received 1000 I.U. HCG intravenously and group IV received 1000 I.U. bovine LH intravenously. All heifers were inseminated artificially at the first estrus following the hormonal treatments. In order to accurately determine the amount of follicular stimulation and ovulation rate, a high lumbar laparotomy was performed on all heifers.

In comparing treatments II and III the amount of stimulation (number of follicles >10 mm. in diameter) and the amount of ovulation for both treatments was found to be quite variable. The mean number of follicles > 10 mm. (including ovulations) for group II was 25.5 compared to 13.6 for group III. Likewise the mean ovulation rate was 7.37 and 5.31, respectively. The number of ovulations as a percentage of the total number of follicles > 10 mm. was found to be 31.9 for group II and 34.7 for group III which was quite low when compared to 52.7 and 54.3 for group I and IV, respectively.

A trend was observed for a greater number of follicles developed to ovulate as the interval from PMS or FSH injection (day 16) to estrus increased. It seems that at least 4 days are necessary for the proper maturation of the follicle and its ovulation. The day 5 interval provided for the greatest number of follicles $>$10 mm. ovulating (50.6%).

No trend was found in the data to suggest a correlation of either individual body weight or breed with the ovarian response obtained. Ovarian activity was found to favor the right ovary both in the number of follicles developing and the number ovulating.

Twenty-eight percent of the heifers in each PMS group had a silent estrous period following treatment. This compares with 16 percent of the controls and 12 percent of the FSH treated cattle. Unexplained is the almost doubled number of follicles $>$10 mm. found in the PMS treated cattle showing estrus in comparison to those showing silent estrus.

A very low percentage of the cattle treated with PMS were found to be pregnant at the time of slaughter. In group II only 12 percent were pregnant and in group III only 24 percent were found pregnant as compared to 61 percent for the controls and 44 percent for the FSH treated cattle.

The stimulation received with the total PMS dosage indicates that these levels must be considered only as causing an excessive amount of ovarian response when thinking along the lines of inducing twin ovulations in the bovine. No significant findings in support or disfavor of the day 5 injection could be observed with the excessive stimulatory response that was obtained. However, with only a total dosage of 5.5 mg. of FSH injected with the day 5 and day 16 sequence, six heifers were multiply stimulated with a desirable number of follicles $>$ 10 mm. (two to six). It is felt that this treatment sequence justifies further study.

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